MAMLD1 and Differences/Disorders of Sex Development: An Update

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Keywords

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Abstract

MAMLD1 (alias *CXorf6*) was first documented in 2006 as a causative gene of 46,XY differences/disorders of sex development (DSD). *MAMLD1/Mamld1* is expressed in the fetal testis and is predicted to enhance the expression of several Leydig cell-specific genes. To date, hemizygous *MAMLD1* variants have been identified in multiple 46,XY individuals with hypomaskulinized external genitalia. Pathogenic *MAMLD1* variants are likely to cause genital abnormalities at birth and are possibly associated with age-dependent deterioration of testicular function. In addition, some *MAMLD1* variants have been identified in 46,XX individuals with ovarian dysfunction. However, recent studies have raised the possibility that *MAMLD1* variants cause 46,XY DSD and ovarian dysfunction as oligogenic disorders. Unsolved issues regarding *MAMLD1* include the association between *MAMLD1* variants and 46,XX testicular DSD, gene-gene interactions in the development of *MAMLD1*-mediated DSD, and intracellular functions of *MAMLD1*.

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Introduction

MAMLD1 (Mastermind like domain containing 1; alias *CXorf6*, OMIM 300120) on the X chromosome is one of the causative genes for 46,XY differences/disorders of sex development (DSD) [Fukami et al., 2006]. The first *MAMLD1* variants associated with 46,XY DSD were reported in 2006 [Fukami et al., 2006]. Subsequently, rare *MAMLD1* variants were identified in several patients with 46,XY DSD and in a few patients with ovarian dysfunction [Kalfa et al., 2008, 2012; Chen et al., 2010; Brandão et al., 2011; Gaspari et al., 2011; Metwalley and Farghaly, 2012; Camats et al., 2015; Igarashi et al., 2015; Eggers et al., 2016; Ratan et al., 2016; Flück et al., 2019; Hughes et al., 2019; Li et al., 2020b]. *MAMLD1* is assumed to play an important role in testicular function during the fetal period and after birth [Fukami et al., 2006; Kalfa et al., 2008, 2012; Camats et al., 2015; Igarashi et al., 2015; Fujisawa et al., 2016]. However, recent studies have shown that some *MAMLD1* variants are shared by unaffected individuals. It is possible that *MAMLD1* variants lead to DSD in combination with other genetic abnormalities [Flück et al., 2019; Camats et al., 2020; Li et al., 2020a]. This review article aims to introduce the current

understanding of biological functions of MAMLD1 in sex development. We also refer to the results of animal studies on *Mamld1*.

The MAMLD1/*Mamld1* Gene

Chromosomal Position and Tissue Expression of MAMLD1/Mamld1

MAMLD1 is located at Xq28, adjacent to *MTM1* that represents a causative gene for myotubular myopathy [Tsai et al., 2005]. The etiological link between *MAMLD1* and 46,XY DSD was first suggested by the phenotype of patients with Xq28 microdeletions. Genetic male patients with these deletions frequently showed hypomaskulinized external genitalia in addition to myopathy [Laporte et al., 1997; Ogata et al., 2008]. *MAMLD1* was the sole gene located within the smallest region of overlap in 6 patients. *MAMLD1* has no homolog on the Y chromosome, and its hemizygous pathogenic variants cause 46,XY DSD in an X-linked recessive fashion. In 46,XX individuals, *MAMLD1* is predicted to undergo X chromosome inactivation [Nazor et al., 2012]. *MAMLD1* is predominantly expressed in human gonads and is variably expressed in most other tissues, except for peripheral leukocytes (GTEx database). In particular, it is strongly expressed in the fetal testis [Fukami et al., 2006].

Murine *Mamld1* is also located on the X chromosome [Miyado et al., 2015]. In situ hybridization analysis of mouse tissues revealed that *Mamld1* is clearly expressed in the Sertoli cells and in most of the Leydig cells of fetal testis [Fukami et al., 2006]. Quantitative PCR revealed that *Mamld1* mRNA expression in the testis gradually increased from 12.5 to 18.5 days post coitum (dpc) [Miyado et al., 2012]. The expression pattern of *Mamld1* in fetal testicular tissues paralleled the increase in testosterone concentration. These findings support the possible role of MAMLD1 in testosterone biosynthesis. In situ hybridization showed that *Mamld1* expression in the postnatal testis became relatively weak, while it was strongly expressed in the granulosa cells of the postnatal ovary [Fukami et al., 2006].

The expression of *MAMLD1* in the testis is likely to be regulated by NR5A1 (alias SF-1/Ad4BP), a key molecule for testicular and adrenal development [Camats et al., 2020]. This notion is based on the previous findings that the *MAMLD1* upstream region harbors a consensus binding motif of NR5A1 and that NR5A1 protein can bind to the sequence and exerts transactivation function [Fukami et al., 2008]. Baetens et al. [2017] have reported that *MAMLD1* was upregulated in lymphocytes of patients with 46,XX tes-

ticular/ovotesticular DSD due to the p.Arg92Trp variant of NR5A1. These data strongly suggest regulatory effects of NR5A1 on *MAMLD1* expression. Other upstream regulators of *MAMLD1* remain to be determined.

Human MAMLD1 Protein Structure

The full-length MAMLD1 protein consists of 774 amino acids (NM_005491.4; NP_005482.2), while a naturally occurring splice variant lacking exon 4 (Δ exon 4) creates a variant protein of 733 amino acids [Fukami et al., 2008]. The MAMLD1 proteins have structural similarity with the MAML2 (Mastermind like 2) protein, a non-DNA binding regulator of the Notch signaling [Fukami et al., 2008]. Both MAMLD1 and MAML2 contain a mastermind like motif and are capable of activating the promoter of *Hes* genes, particularly *Hes3*, in vitro. The full-length and Δ exon 4 forms of *MAMLD1* exert the same in vitro transactivating activity on *Hes* promoters [Fukami et al., 2008]. Since *Hes3* is involved in the regulation of cell differentiation and proliferation [Kageyama et al., 2005], this supports the possible role of MAMLD1 in embryonic development.

In Vitro Function of Mamld1

In vitro knockdown assays of *Mamld1* were performed using mouse Leydig tumor cells (MLTC1) [Nakamura et al., 2011]. *Mamld1* knockdown by 2 siRNAs significantly reduced the mRNA expression of *Cyp17a1* and other genes involved in testosterone production. Consequently, the concentrations of testosterone, androstenedione, and dehydroepiandrosterone were significantly reduced in the culture media of *Mamld1* knockdown cells. In particular, these cells exhibited low testosterone concentration in the medium after 48 h incubation and after 1 h stimulation with human chorionic gonadotropin (hCG). Notably, the expression of *Insl3* involved in testicular descent was also reduced, while the expression of *Hes3* remained unchanged. These data imply that *Mamld1* enhances testosterone production in Leydig cells primarily through the transactivation of *Cyp17a1*. Reduced expression of *INSL3* may also contribute to 46,XY DSD. The results of these knockdown assays indicate that in vivo function of *Mamld1* is likely to be mediated independently of the *Hes3*-related Notch signaling pathway.

Phenotypes of *Mamld1* Knockout Mice

Mamld1 Knockout 40,XY Mice

Miyado et al. [2012] created knockout mice of *Mamld1*. At birth, *Mamld1* knockout 40,XY mice were healthy and

exhibited a typical male genital appearance. Moreover, they showed no abnormalities in testicular histology, testicular weight, or immunohistochemical findings of several marker molecules in the testis. In addition, intra-testicular concentrations of testosterone and other steroids at 18.5 dpc were as expected in normal males. On the other hand, fetal testes of the knockout mice were characterized by low mRNA levels of Leydig cell-specific genes such as *Star*, *Cyp17a1*, *Hsd3b1*, and *Insl3*, compared to the testes of wildtype mice. These results indicate that murine *Mamld1* is involved in the transactivation of several Leydig cell-specific genes in the fetal testis, although it is dispensable for genital masculinization.

In adulthood, *Mamld1* knockout 40,XY mice exhibited relatively small testes [Miyado et al., 2017]. In addition, reduced seminiferous tubule size, decreased number of proliferating spermatogonia and spermatocytes, and mildly impaired daily sperm production were observed. However, these mice exhibited no abnormalities in androgen levels in the serum or testicular tissues. Moreover, the number of pups born from cross-mated wildtype 40,XX mice was comparable between the knockout and wildtype 40,XY mice.

Altogether, these results imply that, in mice, MAMLD1 likely plays supporting roles in multiple steps of male sex development and reproduction. In particular, MAMLD1 appears to upregulate several genes in fetal Leydig cells. The lack of genital abnormalities in *Mamld1* knockout mice indicates that there may be a difference in the functional importance of MAMLD1 between humans and mice. In this regard, although an *Mamld1* variant has been identified in a horse with hypospadias, the pathogenicity of this variant remains uncertain [De Lorenzi et al., 2010]. In addition, *Mamld1* variants were similarly detected in genetic male dogs with and without DSD [Switonski et al., 2012]. Thus, further studies are necessary to clarify whether *Mamld1* variants affect male sex development in non-human species.

Mamld1 Knockout 40,XX Mice

Miyado et al. [2015] also examined the phenotype of *Mamld1* knockout 40,XX mice. Although these mice were apparently healthy, they frequently exhibited parturition failure and resultant death of the pups. This phenotype can be ascribed to defective functional luteolysis. It is known that, in mice, the onset of parturition is triggered by a rapid decline in the blood level of progesterone due to functional luteolysis. The process of functional luteolysis depends on the enzymatic activity of 20 α -hydroxysteroid dehydrogenase (20 α -HSD) in the

corpus luteum. MAMLD1 appears to be involved in the transactivation of *Akr1c18*, the gene encoding 20 α -HSD, because ovaries of *Mamld1* knockout 40,XX mice at late gestation were characterized by low expression levels of *Akr1c18*. Notably, *Stat5b*, a suppressor of *Akr1c18*, was significantly upregulated. Expression of other genes involved in the luteolytic process, such as *Prlr* and *Oxtr*, was also frequently dysregulated. These results indicate that in mice, MAMLD1 plays an indispensable role in labour initiation. MAMLD1 likely facilitates functional luteolysis by regulating the expression of *Stat5b* and other genes. Although it is known that circulating progesterone produced in luteal cells triggers the onset of parturition in several mammals, it remains unknown whether MAMLD1 is also involved in parturition of any species other than mice.

MAMLD1 Variants in 46,XY Individuals

MAMLD1 Variants Identified in Patients with 46,XY DSD

In 2006, 3 *MAMLD1* nonsense variants, p.Glu197* (previously described as p.Glu124*), p.Gln270* (p.Gln197*), and p.Arg726* (p.Arg653*), were identified in 4 Japanese boys with 46,XY DSD [Fukami et al., 2006]. These boys manifested penoscrotal hypospadias with or without micropenis and cryptorchidism. Subsequently, hemizygous *MAMLD1* variants were identified in patients with various types of 46,XY DSD [Kalfa et al., 2008, 2012; Camats et al., 2015; Igarashi et al., 2015; Flück et al., 2019]. *MAMLD1* variants in the Human Gene Mutation Database include several protein-truncating substitutions and missense variants, as well as in-frame insertions and splice site variants (Fig. 1; Table 1) [Fukami et al., 2006; Kalfa et al., 2008, 2012; Chen et al., 2010; Ogata et al., 2012; Baxter et al., 2015; Camats et al., 2015; Igarashi et al., 2015; Ruiz-Arana et al., 2015; Eggers et al., 2016; Flück et al., 2019; Hughes et al., 2019].

We and other researchers performed in vitro assays to assess the function of *MAMLD1* variants (Table 1). First, subcellular localization analyses were performed using fluorescence-tagged proteins [Fukami et al., 2008]. The results showed that the wildtype MAMLD1 exhibited speckled distribution in the nucleus and co-localized with MAML2. This speckled pattern was also observed for the p.Arg726* (p.Arg653*) variant, but not for the p.Glu197* (p.Glu124*) and p.Gln270* (p.Gln197*) variants. Second, luciferase assays using reporter vectors containing the promoters of *Hes3*, *Hes7*, or *CYP17A1* were performed to

Fig. 1. Genomic structure of *MAMLD1*. **a** Structures of the human *MAMLD1* gene (NM_005491.4 and NM_001177465.3) and its protein. The black and white boxes indicate the coding and noncoding exons, respectively. **b** Schematic representation of *MAMLD1* variants described in Table 1. Solid and dashed arrows indicate the variants on NM_005491.4 and NM_001177465.3, respectively. The sizes of introns are not drawn to scale.

examine in vitro transactivating activities of *MAMLD1* variants [Brandão et al., 2011; Camats et al., 2015]. The results showed compromised transactivating activities of several variants including a splice site variant. The Δ exon 4 variant was shown to retain normal activity. In this regard, some *MAMLD1* variants identified in patients with 46,XY DSD showed normal in vitro transactivating activity on the *Hes3* promoter [Fukami et al., 2008; Kalfa et al., 2011; Li et al., 2020b]. Thus, it remains unknown whether these variants are pathogenic mutations or benign polymorphisms. Indeed, these variants are classified into various functional categories according to the criteria of the American College of Medical Genetics and Genomics and the Association for Molecular Pathology [Richards et al., 2015] (Table 1). Third, protein expression analysis revealed reduced expression levels of various nonsense and splice site variants [Fukami et al., 2008; Camats et al., 2015; Igarashi et al., 2015]. Lastly, in silico analyses were

performed to predict the effects of nucleotide substitutions on the 3-dimensional (3D) structure of the *MAMLD1* protein [Kalfa et al., 2011]. These analyses predicted that some missense variants such as p.Pro567Ser (p.Pro542Ser) and p.Ser662Arg (p.Ser589Arg) lead to drastic changes in the interaction force of the amino acid chain and flexibility of the spatial structure.

Phenotypes of Variant-Positive 46,XY Patients

Hemizygous *MAMLD1* variants have been identified in 46,XY individuals with micropenis, cryptorchidism, or complete gonadal dysgenesis [Fukami et al., 2006; Kalfa et al., 2008, 2012; Camats et al., 2015; Igarashi et al., 2015; Flück et al., 2019; Li et al., 2020b]. Li et al. [2020b] performed a review of their own 10 cases and previously reported 26 cases with 46,XY DSD and rare *MAMLD1* variants. The authors analyzed phenotypes of the patients and concluded that hypospadias is the most salient feature of

Table 1. Summary of reported patients harboring *MAMLD1* variants

Patient		MAMLD1 variant				Combination with rare variants in other genes				Reference		
Phenotype	Sex of rearing	hCG test (age at exam, yr:mo)	Müllerian duct derivatives	cDNA (NM_005491.4)	Protein	cDNA (variants reported in the original paper)	Protein	Zygosity	ACMG/AMP classification	In vitro assay		
46,XY												
Partial gonadal dysgenesis	No data	No data	No	c.209A>G ^a	p.Gln70Arg	c.134A>G (NM_001177466)	p.Gln45Arg	Hemizygous	VUS	No data	Eggers et al. [2016]	
	Male	No data	No	c.428C>A ^a	p.Ser143*	c.428C>A (NM_005491)	p.Ser143*	Hemizygous	P	No transactivation function	Kalfa et al. [2012]	
Hypospadias	Male	Normal response (no data)	No	c.445C>T	p.Gln149*	c.370C>T (NM_001177465.3)	p.Gln124*	Hemizygous	LP (P) ^d	No data	Li et al. [2020b]	
Hypospadias	Male	No data	No	c.529C>T	p.Gln177*	c.454C>T (NM_001177465.3)	p.Gln152*	Hemizygous	LP (P) ^d	No data	Li et al. [2020b]	
Hypospadias	Male	No data	No	c.544delG ^a	p.Glu182Serfs*194	c.325delG (NM_005491.1)	p.Glu109Serfs*121	Hemizygous	LP	No data	Kalfa et al. [2008]	
Hypospadias	Male	No data	No	c.544delG ^a	p.Glu182Serfs*194	c.325delG (NM_005491.1)	p.Glu109Serfs*121	Hemizygous	LP	No data	Kalfa et al. [2008]	
Hypospadias	Male	Normal response (0:03)	No	c.544delG ^a	p.Glu182Serfs*194	c.325delG (NM_005491.1)	p.Glu109Serfs*121	Hemizygous	LP	No data	Metvalley and Farhaly [2012]	
Hypospadias	Male	Normal response (2:05) and low response (13:11)	No	c.589G>T ^a	p.Glu197*	c.370G>T (NM_005491.1)	p.Glu124*	Hemizygous	P	No transactivation function	Fukami et al. [2006, 2008]; Fujisawa et al. [2016]	
Hypospadias	Male	Low response (7:00)	No	c.589G>T ^a	p.Glu197*	c.370G>T (NM_005491.1)	p.Glu124*	Hemizygous	P	No transactivation function	Fukami et al. [2006, 2008]; Fujisawa et al. [2016]	
Hypospadias	Male	No data	No	c.605C>T ^a	p.Thr202Met	c.605C>T (NM_005491)	p.Thr202Met	Hemizygous	B	Normal	Camats et al. [2015]	
Hypospadias	Female	Normal response (10 days)	No	c.626delT ^a and c.631G>A	p.Leu210* and p.Asp211Asn	c.626delT and c.631G>A (NM_005491)	p.Leu210* and p.Asp211Asn	Hemizygous	P and LB	Reduced transactivation function and normal	Abnormal GGN repeat in AR	Camats et al. [2015]
(46,XY) DSD	Female	No data	No	c.728G>A ^a	p.Cys243Tyr	c.728G>A (NM_005491)	p.Cys243Tyr	Hemizygous	VUS	No data	Yes	Hughes et al. [2019]
Hypospadias	Male	No data	No	c.808C>T ^a	p.Gln270*	c.589C>T (NM_005491.1)	p.Gln197*	Hemizygous	P	No transactivation function	No data	Fukami et al. [2006, 2008]
Hypospadias	Male	No data	No	c.895C>G	p.Pro299Ala	c.895C>G (NM_005491.4)	p.Pro299Ala	Hemizygous	VUS	No data	No data	Ratan et al. [2016]
Hypospadias	Male	No data	No	c.1041C>A ^a	p.His347Gln	c.1041C>A (NM_005491.4)	p.His347Gln	Hemizygous	B	No data	No data	Kalfa et al. [2012]

Table 1 (continued)

Patient	Phenotype	Sex of rearing	hCG test (age at exam, y/mo)	Müllerian duct derivatives	MAMLD1 variant		Protein	cDNA (variants reported in the original paper)	Protein	Zygosity	ACMG/AMP classification	In vitro assay	Combination with rare variants in other genes	Reference
					cDNA (NM_005491.4)									
Hypospadias	Male		Normal response (no data)	No	c.1041C>A ^a		p.His347Gln	c.1041C>A (NM_005491.4)	p.His347Gln	Hemizygous	B	Normal	Yes	Camats et al. [2015]; Flück et al. [2019]; Camats et al. [2020]
Hypospadias	Female		Normal response (2:00)	No	c.1041C>A ^a		p.His347Gln	c.1041C>A (NM_005491.4)	p.His347Gln	Hemizygous	B	Normal	Yes	Camats et al. [2015]; Flück et al. [2019]; Camats et al. [2020]
Hypospadias	Male		No data	No	c.1075C>T		p.Pro359Ser	c.856C> (NM_005491.1)	p.Pro386Ser	Hemizygous	B	Normal	No data	Fukami et al. [2006, 2008]
Hypospadias	Male		No data	No	c.1075C>T		p.Pro359Ser	c.856C>T (NM_005491.1)	p.Pro386Ser	Hemizygous	B	No data	No data	Chen et al. [2010]
Hypospadias	Male		No data	No	c.1075C>T		p.Pro359Ser	c.856C>T (NM_005491.1)	p.Pro386Ser	Hemizygous	B	Normal	No data	Kalfa et al. [2011]
Hypospadias	Male		No data	No	c.1075C>T		p.Pro359Ser	c.1075C>T (NM_005491)	p.Pro359Ser	Hemizygous	B	No data	No data	Kalfa et al. [2012]
Hypospadias	Male		No data	No	c.1075C>T		p.Pro359Ser	c.1075C>T (NM_005491)	p.Pro359Ser	Hemizygous	B	Normal	No data	Camats et al. [2015]
Hypospadias	Male		Low response (no data)	No	c.1075C>T		p.Pro359Ser	c.1000C>T (NM_001177465.3)	p.Pro334Ser	Hemizygous	B (LP) ^d	No data	Yes	Li et al. [2020b]
Hypospadias	Male		Normal response (no data)	No	c.1075C>T and c.1985A>G		p.Pro359Ser and p.Asn662Ser	c.856C>T and c.1766A>G (NM_005491.1)	p.Pro386Ser and p.Asn589Ser	Hemizygous	B and B	No data	No data	Gaspari et al. [2011]
Hypospadias	Male		No data	No	c.1075C>T and c.1985A>G		p.Pro359Ser and p.Asn662Ser	c.856C>T and c.1766A>G (NM_005491.1)	p.Pro386Ser and p.Asn589Ser	Hemizygous	B and B	Normal	No data	Kalfa et al. [2011]
Hypospadias	Male		No data	No	c.1075C>T and c.1985A>G		p.Pro359Ser and p.Asn662Ser	c.1075C>T and c.1985A>G (NM_005491)	p.Pro359Ser and p.Asn662Ser	Hemizygous	B and B	No data	No data	Kalfa et al. [2012]
46,X,Y DSD	Male		No data	No	c.1141C>T ^a		p.Arg381*	c.1066C>T (NM_001177466)	p.Arg356*	Hemizygous	LP (P) ^d	No data	No data	Eggers et al. [2016]
46,X,Y DSD	Female		No data	No	c.1141C>T ^a		p.Arg381*	c.1066C>T (NM_001177466)	p.Arg356*	Hemizygous	LP (P) ^d	No data	No data	Eggers et al. [2016]
Disorders of androgen synthesis or action	Male		No data	No	c.1141C>T ^a		p.Arg381*	c.1066C>T (NM_001177466)	p.Arg356*	Hemizygous	LP (P) ^d	No data	No data	Eggers et al. [2016]
Hypospadias	Male		Normal response (no data)	No	c.1141C>T ^a		p.Arg381*	c.1066C>T (NM_001177465.3)	p.Arg356*	Hemizygous	LP (P) ^d	No data	Yes	Li et al. [2020b]
Hypospadias	Male		Normal response (no data)	No	c.1141C>T ^a		p.Arg381*	c.1066C>T (NM_001177465.3)	p.Arg356*	Hemizygous	LP (P) ^d	No data	Yes	Li et al. [2020b]

Table 1 (continued)

Patient	MAMLD1 variant				Combination with rare variants in other genes	Reference						
	Phenotype	Sex of rearing	hCG test (age at exam, yr:mo)	Müllerian duct derivatives								
cDNA (NM_005491.4)												
Protein												
cDNA (variants reported in the original paper)												
Protein												
Zygosity												
ACMG/AMP classification												
In vitro assay												
Reduced transactivation function												
Hypospadias	Male	No data	No	c.1151C>T ^a	p.Pro384Leu	c.1151C>T (NM_005491)	p.Pro384Leu	Hemizygous	LP	Reduced transactivation function	No data	Kalfa et al. [2012]
46,XY DSD	Male	No data	No	c.1336G>C	p.Ala446Pro	c.1261G>C (NM_001177465.3)	p.Ala421Pro	Hemizygous	VUS	No data	Yes	Li et al. [2020b]
Hypospadias	Male	No data	No	c.1441C>T	p.Arg481*	c.1366C>T (NM_001177465.3)	p.Arg456*	Hemizygous	LP	No data	Yes	Hughes et al. [2019]
Hypospadias	Male	No data	No	c.1500_1505dupGCAGCA ^a	p.Gln501Gln502	c.1503_1504dupCAGCAG (NM_005491)	p.Gln501Gln502	Hemizygous	VUS	Normal	Yes	Camats et al. [2015]; Flück et al. [2019]; Camats et al. [2020]
46,XY DSD	Male	No data	No	c.1503_1505dupGCA ^a	p.Gln502dup	c.1479_1480dupCAG (NM_005491.3)	p.Gln502dup	Hemizygous	VUS	No data	No data	Baxter et al. [2015]
Hypospadias	Male	Normal response (0:09)	No	c.1508C>A ^a	p.Ala503Glu	c.1508C>A (NM_005491)	p.Ala503Glu	Hemizygous	VUS	Normal	Yes	Camats et al. [2015]; Flück et al. [2019]; Camats et al. [2020]
Hypospadias	Male	No data	No	c.1514T>C ^a	p.Val505Ala	c.1295T>C (NM_005491.1)	p.Val432Ala	Hemizygous	B	No data	No data	Kalfa et al. [2008]; Ogata et al. [2012]
Hypospadias	Male	No data	No	c.1514T>C ^a	p.Val505Ala	c.1295T>C (NM_005491.1)	p.Val432Ala	Hemizygous	B	No data	No data	Chen et al. [2010]
Hypospadias	Female	Normal response (no data)	No	c.1514T>C ^a	p.Val505Ala	c.1514T>C (NM_005491)	p.Val505Ala	Hemizygous	B	Normal	Yes	Camats et al. [2015]; Flück et al. [2019]; Camats et al. [2020]
46,XY DSD	No data	No data	No	c.1570C>T ^a	p.Gln524*	c.1495C>T (NM_001177466)	p.Gln499*	Hemizygous	LP (P) ^d	No data	No data	Eggers et al. [2016]
46,XY DSD	No data	No data	No	c.1699C>T ^a	p.Pro567Ser	c.1624C>T (NM_001177466)	p.Pro542Ser	Hemizygous	B (VUS) ^d	No data	No data	Eggers et al. [2016]
Hypospadias	Male	Normal response (no data)	No	c.1699C>T ^a	p.Pro567Ser	c.1624C>T (NM_001177465.3)	p.Pro542Ser	Hemizygous	B (LB) ^d	No data	Yes	Li et al. [2020b]
Female genitalia	Male	No data	No	c.1739A>G	p.Gln580Arg	c.1520T>C (NM_005491.1)	p.Gln507Arg	Hemizygous	VUS	Normal	No data	Fukami et al. [2006, 2008]
Female genitalia	Male	No data	No	c.1739A>G	p.Gln580Arg	c.1520T>C (NM_005491.1)	p.Gln507Arg	Hemizygous	VUS	Normal	No data	Fukami et al. [2006, 2008]
Hypospadias	Male	No data	No	c.1804C>A ^a	p.Gln602Lys ^b	c.1585C>A (NM_005491.1)	p.Gln529Lys	Hemizygous	B	No data	No data	Chen et al. [2010]
Hypospadias	Male	No data	No	c.1810ins(CAG) ₃ ^a	p.Gln604ins3Gln	CAG ₁₀ →CAG ₁₃ (NM_005491)	p.531ins3Gln	Hemizygous	VUS	No data	No data	Kalfa et al. [2008]
Hypospadias	Male	No data	No	c.1810ins(CAG) ₃ ^a	p.Gln604ins3Gln	c.1591ins(CAG) ₃ (NM_005491.1)	p.531ins3Gln	Hemizygous	VUS	No data	No data	Chen et al. [2010]
46,XY DSD	Male	No data	No	c.1985A>G	p.Asn662Ser	c.1766A>G (NM_005491.1)	p.Asn589Ser	Hemizygous	B	Normal	No data	Fukami et al. [2006, 2008]

Table 1 (continued)

Patient		MAMLD1 variant				Combination with rare variants in other genes				Reference		
Phenotype	Sex of rearing	hCG test (age at exam, yr:mo)	Müllerian duct derivatives	cDNA (NM_005491.4)	Protein	cDNA (variants reported in the original paper)	Protein	Zygosity	ACMG/AMP classification	In vitro assay		
Hypospadias	Male	No data	No	c.1985A>G	p.Asn662Ser	c.1766A>G (NM_005491.1)	p.Asn589Ser	Hemizygous	B	No data	Chen et al. [2010]	
Hypospadias	Male	No data	No	c.1985A>G	p.Asn662Ser	c.1766A>G (NM_005491.1)	p.Asn589Ser	Hemizygous	B	Normal	Kalfa et al. [2011]	
Hypospadias	Male	No data	No	c.1985A>G	p.Asn662Ser	c.1985A>G (NM_005491)	p.Asn662Ser	Hemizygous	B	No data	Kalfa et al. [2012]	
Hypospadias	Male	No data	No	c.1985A>G	p.Asn662Ser	c.1985A>G (NM_005491.4)	p.Asn662Ser	Hemizygous	B	No data	Kon et al. [2015]	
Hypospadias	Male	No data	No	c.1985A>G	p.Asn662Ser	c.1985A>G (NM_005491.4)	p.Asn662Ser	Hemizygous	B	Normal	Camats et al. [2015]	
Gonadal dysgenesis	Female	No data	Yes	c.2030C>T ^a	p.Pro677Leu	c.2030C>T (NM_005491)	p.Pro677Leu	Hemizygous	LP	No transactivation function	Ruiz-Arana et al. [2015]	
Hypospadias	Male	No data	No	c.2041-2A>G ^a		g.IV54-2A>G (NM_001177465)		Hemizygous	P	Translation failure	Igarashi et al. [2015]	
Hypospadias	Male	No data	No	c.2170C>G ^a	p.Leu724Val	c.2170C>G (NM_005491.4)	p.Leu724Val	Hemizygous	VUS	Normal	Yes	Camats et al. [2015]; Flück et al. [2019]; Camats et al. [2020]
Hypospadias	Male	Low response (9:09)	No	c.2176C>T ^a	p.Arg726*	c.1957C>T (NM_005491.1)	p.Arg653*	Hemizygous	P	Normal	No data	Fukami et al. [2006,2008]; Fujisawa et al. [2016]
Hypospadias	Female	No data	Yes	c.2190G>A	p.Ser730Ser	c.2190G>A (NM_005491.4)	p.Ser730Ser	Hemizygous	LB	Normal	Yes	Camats et al. [2015]; Flück et al. [2019]; Camats et al. [2020]
Hypospadias	Male	No data	No	c.2277C>T	p.Asp759Asp	c.2058C>T (NM_005491.1)	p.Asp686Asp	Hemizygous	LB	No data	No data	Chen et al. [2010]
Hypospadias	Male	No data	No	c.2284+8A>T		c.2065+8A>T (NM_005491.1)		Hemizygous	B	No data	No data	Chen et al. [2010]
Hypospadias	Male	Normal response (no data)	No	3' UTR	No	c.1986C>G (NM_001177465.3)	p.Ser662Arg	Hemizygous	VUS	No data	Yes	Li et al. [2020b]
Hypospadias	Male	No data	No	3' UTR	No	c.2025C>A (NM_001177465)	p.Asn675Lys	Hemizygous	VUS	No data	No data	Kon et al. [2015]
46,XY DSD	No data	No data	No	3' UTR	No	c.2486C>T (NM_001177465)	p.Ala829Val	Hemizygous	VUS	No data	No data	Eggers et al. [2016]
(46,XY?) DSD	Female	No data	No	3' UTR	No	c.2744A>C (NM_001177465.3)	p.Asp915Ala	Hemizygous	B	No data	Yes	Hughes et al. [2019]
Hypospadias	Male	No data	No	3' UTR	No	c.2780G>T (NM_001177465.3)	p.Arg927Leu	Hemizygous	B (LB) ^d	No data	Yes	Li et al. [2020b]
Hypospadias	Male	No data	No	3' UTR	No	c.2975C>T (NM_001177465)	p.Thr992Ile	Hemizygous	VUS	No data	No data	Eggers et al. [2016]
46,XY DSD	Male	No data	No	3' UTR	No	c.2975C>T (NM_001177465.3)	p.Thr992Ile	Hemizygous	VUS	No data	Yes	Li et al. [2020b]

Table 1 (continued)

Patient	MAMLD1 variant			Protein	cDNA (variants reported in the original paper)	Protein	Zygosity	ACMG/AMP classification	In vitro assay	Combination with rare variants in other genes	Reference
	Phenotype	Sex of rearing	hCG test (age at exam, yrmo)	Müllerian duct derivatives	cDNA (NM_005491.4)						
46,XX											
46,XX DSD	Female	No response (no data)	No	No	c.1514T>C ^a	p.Val505Ala	Homozygous ^c	B	High transactivation activity	No data	Brandão et al. [2011]

ACMG/AMP, the American College of Medical Genetics and Association of Molecular Pathology; hCG, human chorionic gonadotropin; MAMLD1, Mastermind like domain containing 1; JTR, untranslated region; B, benign; LB, likely benign; P, pathogenic; VUS, variant of uncertain significance. ^a These variants were submitted to the Human Gene Mutation Database (<http://www.hgmd.cf.ac.uk/ac/index.php>). ^b This variant is predicted to disrupt exonic splicing. ^c This variant was submitted to the database as a hemizygous substitution. ^d The score described in the original paper is shown in brackets.

MAMLD1 abnormalities. The severity of hypospadias varied among the patients. In addition, micropenis was observed in about half of the cases and complete female-type external genitalia was present in 4 cases. Adrenal dysfunction was not observed. Rare clinical features observed only in one or a few patients included craniofacial anomalies and precocious puberty. Only 1 patient had müllerian duct derivatives [Ruiz-Arana et al., 2015] (Table 1).

Most of the patients were subjected to blood hormone measurements during infancy. The levels of testosterone and gonadotropins were mostly within the normal range. Likewise, adrenal hormones were normal in all the patients examined. However, blood levels of LH, and FSH were significantly elevated in 1 Chinese patient who exhibited precocious puberty [Li et al., 2020b].

The current understanding is that the major role of *MAMLD1* during the fetal period is to enhance testosterone biosynthesis, and loss-of-function variants of *MAMLD1* typically lead to partial hypomasculinization at birth. However, it remains unknown whether complete female-type external genitalia and precocious puberty identified in some patients are associated with the *MAMLD1* variants. Furthermore, genotype-phenotype correlations of *MAMLD1* variants have yet to be studied.

Long Term Follow-Up Study of Patients with 46,XY DSD and MAMLD1 Variants

Fujisawa et al. [2016] performed a longitudinal follow-up study of 3 patients with 46,XY DSD and hemizygous *MAMLD1* nonsense variants. At birth, these individuals exhibited penoscrotal hypospadias with chordee, micropenis, hypoplastic scrotum, and/or undescended/retractile testis. Blood levels of testosterone during infancy were within the normal range. However, these 3 patients invariably manifested impaired testicular function at later ages. One of the 3 patients (patient 1) manifested incomplete secondary sexual development together with micropenis and relatively small testes. The patient showed an inappropriate response of blood testosterone to the hCG stimulation. The remaining 2 patients (patients 2 and 3) also exhibited borderline micropenis at 7 and 6 years of age, respectively. Blood examinations of patients 2 and 3 at 7 and 9 years of age, respectively, suggested mild to moderate hypergonadotropic hypogonadism. In addition, ultrasound examinations revealed testicular micro-lithiasis, a non-specific sign of testicular dysfunction, in patients 1 and 3. These results indicate that loss-of-function mutations of *MAMLD1* cause not only genital abnormalities at birth, but also age-dependent deterioration of

testicular function. Consistent with this, delayed puberty indicative of partial hypogonadism was observed in a Swiss patient with a *MAMLD1* variant [Li et al., 2020b]. On the other hand, a Chinese patient exhibited early puberty [Li et al., 2020b]. Thus, further studies are necessary to clarify the long term clinical features of patients with *MAMLD1* mutations.

Oligogenicity of MAMLD1 Variants

As mentioned above, *MAMLD1* variants were identified in a number of patients with 46,XY DSD. However, there is a debate about the functional significance of *MAMLD1* in male sex development. Indeed, several findings, such as the identification of some *MAMLD1* variants in the general population, preserved in vitro transactivating activity of several DSD-associated *MAMLD1* variants, phenotypic discrepancies among the patients with the same *MAMLD1* variants, and the typical male genital appearance and preserved fertility of *Mamld1* knockout 40,XY mice, pose a question as to whether single *MAMLD1* variants are sufficient to cause 46,XY DSD [Fukami et al., 2008; Miyado et al., 2012, 2017; Camats et al., 2020].

Flück et al. [2019] raised the possibility that *MAMLD1* variants lead to 46,XY DSD when combined with rare variants in other genes. To test this hypothesis, the authors performed whole exome analysis for 7 patients with 46,XY DSD and 1 patient with 46,XX ovarian insufficiency, who harbored hemizygous and heterozygous *MAMLD1* variants, respectively. The authors analyzed the sequences of 606 genes that were possibly associated with *MAMLD1*-related signaling pathways or fetal sex development. As a result, Flück et al. [2019] identified 55 rare heterozygous/hemizygous variants in 41 genes that potentially contributed to the disease phenotypes of the 8 patients. For example, patient 1 harbored 9 rare nucleotide substitutions in 7 genes, in addition to the p.Val505Ala (p.Val432Ala) variant of *MAMLD1*. Most of the 7 genes have been associated with hypospadias and/or syndromic genital anomalies. The remaining 7 patients also carried 4 or more rare variants in the tested genes. Most of the identified variants were classified into the categories of “variant of unknown significance (VUS)” or “likely-benign”; however, 2 variants were assessed as “likely pathogenic”. These results support the oligogenic nature of *MAMLD1*-associated DSD. Since the first 3 patients with *MAMLD1* variants [Fukami et al., 2006] were not subjected to whole exome analysis, these individuals may also carry pathogenic variants in other genes. In this regard, systematic mutation screening for patients with

46,XY DSD have shown that a certain percentage of the patients harbored multiple variants in known causative genes [Kon et al., 2015; Eggers et al., 2016]. Thus, although there is no doubt that monogenic mutations account for a certain percentage of cases with 46,XY DSD, oligogenicity is likely to play an important role in the etiology of this disorder.

MAMLD1 Variants in 46,XX Individuals

Thus far, there are only a few reports of *MAMLD1* variants in genetic females [Flück et al., 2019]. 46,XX individuals with heterozygous loss-of-function variants of *MAMLD1* appear to be phenotypically unremarkable, because the mothers of patients with 46,XY DSD and *MAMLD1* variants were reported to be healthy and fertile [Fukami et al., 2006; Kalfa et al., 2012; Ruiz-Arana et al., 2015]. However, considering the strong expression of *MAMLD1/Mamld1* in the postnatal ovaries of humans and mice [Fukami et al., 2006; Miyado et al., 2015], *MAMLD1* may play a role in ovarian function. Consistent with this, Flück et al. [2019] reported a 27-year-old Spanish woman with primary amenorrhea who carried a rare heterozygous variant in *MAMLD1*. The woman exhibited small ovaries with a large number of primordial follicles and atresic follicles. Endocrine examination confirmed hypergonadotropic hypogonadism. These data indicate that *MAMLD1* possibly contributes to ovarian development or function. This notion is further supported by the report by Marozzi et al. [2000], in which the authors identified chromosomal deletion at Xq28 in a female with premature ovarian failure. In this regard, because the woman reported by Flück et al. [2019] harbored 14 additional variants in genes possibly associated with *MAMLD1*-related signaling pathways or fetal sex development, heterozygous *MAMLD1* variants may cause ovarian dysfunction as an oligogenic disorder. Thus far, there have been no reports of homozygous loss-of-function variants of *MAMLD1* in women. Thus, although homozygous deletion of *Mamld1* was shown to cause parturition failure in 40,XX mice [Miyado et al., 2015], it remains unknown whether complete loss-of-function of *MAMLD1* also leads to delayed delivery in humans.

In addition, Brandão et al. [2011] reported a 44-year-old woman with bilateral streak gonads and moderate clitoromegaly, in whom the authors identified a homozygous missense variant p.Val505Ala (p.Val432Ala) of *MAMLD1*. The patient was born to consanguineous parents. The variant *MAMLD1* protein exerted higher in vi-

tro transactivating activity for *Hes3* and *Hes7* promoters than the wildtype protein. These results provide the first indication that gain-of-function variants of *MAMLD1* may be associated with ovarian dysfunction with partial masculinization. In this context, Baetens et al. [2017] reported that *MAMLD1* is upregulated in the lymphocytes of individuals with 46,XX testicular DSD due to the p.Arg92Trp variant of *NR5A1*. These results imply that increased *MAMLD1* expression contributes to testicular development in genetic females. However, because no cases with 46,XX testicular DSD and pathogenic *MAMLD1* variants have been reported so far, this speculation awaits further validation.

Further Perspectives

MAMLD1 Function

The role of *MAMLD1* in sex development is not fully understood. For example, it remains unknown how *MAMLD1* transactivates its target genes without binding to DNA. Moreover, in vitro assays suggested that the function of *MAMLD1* to promote testicular development is independent of *Hes3* [Fukami et al., 2008]. Future studies on the oligogenicity of *MAMLD1*-related DSD may provide a clue to understand the gene-gene interactions involving *MAMLD1* in the developing gonads.

MAMLD1 Intracellular Localization

Although *MAMLD1* was shown to reside in the nucleus in a speckled pattern [Fukami et al., 2008], the organelle which involves *MAMLD1* has yet to be determined. In this regard, recent studies have demonstrated that mammalian cells contain a number of membraneless organelles both in the nucleus and in the cytoplasm [Hyman et al., 2014; Banani et al., 2017]. These membraneless organelles are created through liquid-liquid phase separation (LLPS) and facilitate various biochemical reactions including transcriptional regulation and RNA modification. In particular, recent studies have suggested that SOX9 is located in the paraspeckle, one of the major LLPS-associated nuclear organelles, and transactivates its target genes [Hata et al., 2008; Akiba et al., 2020]. These data indicate that LLPS contributes to testicular development by providing a functional platform for SOX9. The distribution pattern of *MAMLD1* implies that it may also reside within an LLPS-associated membraneless organelle.

Conclusions

A number of rare *MAMLD1* variants have been identified in patients with 46,XY DSD. These patients typically exhibited hypospadias indicative of subnormal androgen production/function during the embryonic period. In addition, some *MAMLD1* variants have been linked to ovarian insufficiency in women. However, recent studies have raised the possibility that *MAMLD1* variants cause 46,XY DSD and ovarian dysfunction as oligogenic disorders. Unsolved issues regarding *MAMLD1* include the association between its variants and 46,XX testicular DSD and gene-gene interactions in the development of *MAMLD1*-mediated DSD.

Conflict of Interest Statement

The authors have nothing to disclose.

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Author Contribution

M.F. drafted the manuscript. M.M. edited the text and prepared the figure and the table. T.O. edited the manuscript. All authors reviewed the final version of the manuscript.

References

- Akiba K, Narumi S, Nishimura R, Kato-Fukui Y, Takada S, Hasegawa Y, et al. SOX9 is colocalized with paraspeckle protein NONO in cultured murine sertoli cells and features structural characteristics of intrinsically disordered proteins. *Mol Reprod Dev.* 2020;87:1124–5.
- Baetens D, Stoop H, Peelman F, Todeschini AL, Rosseel T, Coppieters F, et al. NR5A1 is a novel disease gene for 46,XX testicular and ovotesticular disorders of sex development. *Genet Med.* 2017;19:367–76.
- Banani SF, Lee HO, Hyman AA, Rosen MK. Biomolecular condensates: organizers of cellular biochemistry. *Nat Rev Mol Cell Biol.* 2017;18:285–98.
- Baxter RM, Arboleda VA, Lee H, Barseghyan H, Adam MP, Fechner PY, et al. Exome sequencing for the diagnosis of 46,XY disorders of sex development. *J Clin Endocrinol Metab.* 2015;100:E333–44.

- Brandão MP, Costa EM, Fukami M, Gerdulo M, Pereira NP, Domenice S, et al. MAMLD1 (mastermind-like domain containing 1) homozygous gain-of-function missense mutation causing 46,XX disorder of sex development in a virilized female. *Adv Exp Med Biol*. 2011;707:129–31.
- Camats N, Fernández-Cancio M, Audí L, Mullis PE, Moreno F, González Casado I, et al. Human MAMLD1 gene variations seem not sufficient to explain a 46,XY DSD phenotype. *PLoS One*. 2015;10:e0142831.
- Camats N, Flück CE, Audí L. Oligogenic origin of differences of sex development in humans. *Int J Mol Sci*. 2020;21:1809.
- Chen Y, Thai HT, Lundin J, Lagerstedt-Robinson K, Zhao S, Markljung E, et al. Mutational study of the MAMLD1-gene in hypospadias. *Eur J Med Genet*. 2010;53:122–6.
- De Lorenzi L, Genuardo V, Iannuzzi A, Di Meo GP, Perucatti A, Mancuso R, et al. Cytogenetic and genetic studies in a hypospadiac horse (*Equus caballus*, 2n = 64). *Sex Dev*. 2010;4:352–7.
- Eggers S, Sadedin S, van den Bergen JA, Robevska G, Ohnesorg T, Hewitt J, et al. Disorders of sex development: insights from targeted gene sequencing of a large international patient cohort. *Genome Biol*. 2016;17:243.
- Flück CE, Audí L, Fernández-Cancio M, Sauter KS, Martínez de LaPiscina I, Castaño L, et al. Broad phenotypes of disorders/differences of sex development in MAMLD1 patients through oligogenic disease. *Front Genet*. 2019;10:746.
- Fujisawa Y, Fukami M, Hasegawa T, Uematsu A, Muroya K, Ogata T. Long-term clinical course in three patients with MAMLD1 mutations. *Endocr J*. 2016;63:835–9.
- Fukami M, Wada Y, Miyabayashi K, Nishino I, Hasegawa T, Nordenskjöld A, et al. CXorf6 is a causative gene for hypospadias. *Nat Genet*. 2006;38:1369–71.
- Fukami M, Wada Y, Okada M, Kato F, Katsumata N, Baba T, et al. Mastermind-like domain-containing 1 (MAMLD1 or CXorf6) transactivates the Hes3 promoter, augments testosterone production, and contains the SF1 target sequence. *J Biol Chem*. 2008;283:5525–32.
- Gaspari L, Paris F, Philibert P, Audran F, Orsini M, Servant N, et al. Idiopathic partial androgen insensitivity syndrome in 28 newborn and infant males: impact of prenatal exposure to environmental endocrine disruptor chemicals? *Eur J Endocrinol*. 2011;165:579–87. <https://doi.org/10.1530/EJE-11-0580>.
- Hata K, Nishimura R, Muramatsu S, Matsuda A, Matsubara T, Amano K, et al. Paraspeckle protein p54nrb links Sox9-mediated transcription with RNA processing during chondrogenesis in mice. *J Clin Invest*. 2008;118:3098–108.
- Hughes LA, McKay-Bounford K, Webb EA, Dasani P, Clokie S, Chandran H, et al. Next-generation sequencing (NGS) to improve the diagnosis and management of patients with disorders of sex development (DSD). *Endocr Connect*. 2019;8:100–10.
- Hyman AA, Weber CA, Jülicher F. Liquid-liquid phase separation in biology. *Annu Rev Cell Dev Biol*. 2014;30:39–58.
- Igarashi M, Wada Y, Kojima Y, Miyado M, Nakamura M, Muroya K, et al. Novel splice site mutation in MAMLD1 in a patient with hypospadias. *Sex Dev*. 2015;9:130–5.
- Kageyama R, Ohtsuka T, Hatakeyama J, Ohsawa R. Roles of bHLH genes in neural stem cell differentiation. *Exp Cell Res*. 2005;306:343–8.
- Kalfa N, Liu B, Klein O, Ophir K, Audran F, Wang MH, et al. Mutations of CXorf6 are associated with a range of severities of hypospadias. *Eur J Endocrinol*. 2008;159:453–8.
- Kalfa N, Cassorla F, Audran F, Oulad Abdennabi I, Philibert P, Bérout C, et al. Polymorphisms of MAMLD1 gene in hypospadias. *J Pediatr Urol*. 2011;7:585–91.
- Kalfa N, Fukami M, Philibert P, Audran F, Pienkowski C, Weill J, et al. Screening of MAMLD1 mutations in 70 children with 46,XY DSD: identification and functional analysis of two new mutations. *PLoS One*. 2012;7:e32505.
- Kon M, Suzuki E, Dung VC, Hasegawa Y, Mitsui T, Muroya K, et al. Molecular basis of non-syndromic hypospadias: systematic mutation screening and genome-wide copy-number analysis of 62 patients. *Hum Reprod*. 2015;30:499–506.
- Laporte J, Guiraud-Chaumeil C, Vincent MC, Mandel JL, Tanner SM, Liechti-Gallati S, et al. Mutations in the MTM1 gene implicated in X-linked myotubular myopathy. ENMC International Consortium on Myotubular Myopathy. European Neuro-Muscular Center. *Hum Mol Genet*. 1997;6:1505–11.
- Li L, Gao F, Fan L, Su C, Liang X, Gong C. Disorders of sex development in individuals harbouring MAMLD1 variants: WES and interactome evidence of oligogenic inheritance. *Front Endocrinol*. 2020a;11:582516.
- Li L, Su C, Fan L, Gao F, Liang X, Gong C. Clinical and molecular spectrum of 46,XY disorders of sex development that harbour MAMLD1 variations: case series and review of literature. *Orphanet J Rare Dis*. 2020b;15:188.
- Marozzi A, Manfredini E, Tibiletti MG, Furlan D, Villa N, Vegetti W, et al. Molecular definition of Xq common-deleted region in patients affected by premature ovarian failure. *Hum Genet*. 2000;107:304–11.
- Metwalley KA, Farghaly HS. X-linked congenital adrenal hypoplasia associated with hypospadias in an Egyptian baby: a case report. *J Med Case Rep*. 2012;6:428.
- Miyado M, Nakamura M, Miyado K, Morohashi K, Sano S, Nagata E, et al. Mamld1 deficiency significantly reduces mRNA expression levels of multiple genes expressed in mouse fetal Leydig cells but permits normal genital and reproductive development. *Endocrinology*. 2012;153:6033–40.
- Miyado M, Miyado K, Katsumi M, Saito K, Nakamura A, Shihara D, et al. Parturition failure in mice lacking Mamld1. *Sci Rep*. 2015;5:14705.
- Miyado M, Yoshida K, Miyado K, Katsumi M, Saito K, Nakamura S, et al. Knockout of murine Mamld1 impairs testicular growth and daily sperm production but permits normal postnatal androgen production and fertility. *Int J Mol Sci*. 2017;18:1300.
- Nakamura M, Fukami M, Sugawa F, Miyado M, Nonomura K, Ogata T. Mamld1 knockdown reduces testosterone production and Cyp17a1 expression in mouse Leydig tumor cells. *PLoS One*. 2011;6:e19123.
- Nazor KL, Altun G, Lynch C, Tran H, Harness JV, Slavin I, et al. Recurrent variations in DNA methylation in human pluripotent stem cells and their differentiated derivatives. *Cell Stem Cell*. 2012;10:620–34.
- Ogata T, Fukami M, Wada Y. MAMLD1 (CXorf6) is a new gene for hypospadias. *Clin Pediatr Endocrinol*. 2008;17:87–93.
- Ogata T, Sano S, Nagata E, Kato F, Fukami M. MAMLD1 and 46,XY disorders of sex development. *Semin Reprod Med*. 2012;30:410–6.
- Ratan SK, Sharma A, Kapoor S, Polipalli SK, Dubey D, Mishra TK, et al. Polymorphism of 3' UTR of MAMLD1 gene is also associated with increased risk of isolated hypospadias in Indian children: a preliminary report. *Pediatr Surg Int*. 2016;32:515–24.
- Richards S, Aziz N, Bale S, Bick D, Das S, Gastier-Foster J, et al. Standards and guidelines for the interpretation of sequence variants: a joint consensus recommendation of the American College of Medical Genetics and Genomics and the Association for Molecular Pathology. *Genet Med*. 2015;17:405–24.
- Ruiz-Arana IL, Hübner A, Cetingdag C, Krude H, Grüters A, Fukami M, et al. A novel hemizygous mutation of MAMLD1 in a patient with 46,XY complete gonadal dysgenesis. *Sex Dev*. 2015;9:80–5.
- Switonski M, Payan-Carreira R, Bartz M, Nowacka-Woszuk J, Szczerbal I, Colaço B, et al. Hypospadias in a male (48,XY; SRY-positive) dog and sex reversal female (48,XX; SRY-negative) dogs: clinical, histological and genetic studies. *Sex Dev*. 2012;6:128–34.
- Tsai TC, Horinouchi H, Noguchi S, Minami N, Murayama K, Hayashi YK, et al. Characterization of MTM1 mutations in 31 Japanese families with myotubular myopathy, including a patient carrying 240 kb deletion in Xq28 without male hypogonadism. *Neuromuscul Disord*. 2005;15:245–52.